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13. ABSTRACT (Maximum 200 Words) Expression constructs have been prepared in order to generate the 89-143 fragment of the prion protein with isotopic labels using either <i>in vitro</i> translation or expression in <i>E.coli</i> cells. Initial testing of expression has been done. Conditions have been investigated for rapidly dissolving fibrils of PrP(89-143) and proteolytically fragmenting them for mass spectroscopic analysis to probe the extent of hydrogen exchange of backbone amides with deuterated water solvent. Conditions for complementary NMR analysis using organic solvent to dissolve the fibrils have also been investigated in a related model system, TTR. Solid state NMR measurements have been done with synthesized PrP(89-143) peptides incorporating specific ¹³ C labels. These measurements probe the conformation around glycine residues in addition to several other backbone sites in the segment from residue 112 to 123. These measurements indicate that residues in this section of PrP(89-143) are primarily extended beta structure in fibrils, though G113 may be less ordered or part of a turn.				
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INTRODUCTION

The goals of this project are to prepare samples of peptide 89-143 from the mouse prion protein in new ways to facilitate isotope labeling for use in solid state NMR, and to carry out solid state NMR studies of the conformation of this peptide. In addition measurements of the exchange of deuterium for hydrogen in the backbone amides of this peptide in fibrils are being done to define sites of hydrogen bonding, a complementary constraint on the conformation, and to probe whether there are different conformations in different forms of PrP. Ultimately these studies are to be extended to intact PrP to understand its conformation, and how this may relate to disease associated activity; and to understand whether there is a conformational basis for 'strains' of 'scrapie' PrP. The validity of this peptide and its induction of disease as a model for Scrapie diseases has recently received further support [1].

BODY

During the past year we have made peptide samples by chemical synthesis to enable some immediate solid state NMR experiments, described further in a later paragraph. We have also worked on peptide preparation using biological synthesis. To initiate this we constructed a gene to express the 89-143 peptide carrying the P101L mutation, which we will designate here P₅₅ (55 residue peptide). In doing this, the choice of codons could be made typical for *E.coli*, which should facilitate bacterial expression. The constructed gene was prepared in a cloning vector. From this, using PCR, we extracted the segment coding for P₅₅ with restriction sites for insertion into other vectors (a minor error in primer selection slowed this process). For *in vitro* expression we ligated this fragment into pIVEX (a vector optimized by Roche for *in vitro* translation, containing a histidine tag to facilitate purification), and have begun testing production. Although we have gotten good expression of a GFP control, to date we have not seen high production of P₅₅ with the N-terminal his₆ tag. We are now testing for proper mRNA production and stability, and are in the process of cloning into other versions of pIVEX (C-terminal tag or no tag). In addition to pIVEX we also cloned the gene for P₅₅ into standard T7 driven bacterial expression vectors, which encode just the peptide, or the peptide expressed as a fusion with the TrpE leader (an insoluble product that goes into inclusion bodies), and alternately with GST (a soluble, affinity purifiable tag) or MBP (a large very soluble, affinity purifiable tag). All of these have been tested, and express in *E.coli* cells at reasonable levels. Conditions are being optimized for expression (we want to maximize production on the relatively expensive isotope enriched medium), and we are starting to work out purification protocols.

P₅₅ was also made by direct chemical synthesis without isotope labels and purified by HPLC, to initiate the hydrogen exchange by mass spectroscopy experiments. Fibrils were grown in the manner described by Kaneko et al. [2]. Students in the lab were trained in electron microscopy so that we could directly verify fibril formation. In order to analyze the extent of hydrogen exchange in different segments of the peptide we need to be able to fragment P₅₅ using proteases. To this end, conditions were screened to rapidly dissolve fibrils under conditions that quench exchange (0 °C, pH 2.5), retaining information about exchange in the fibril state and still be compatible with peptic digests and MALDI without any intermediary chromatographic step. The optimized dissolution conditions were 8% TFA, followed by a ten fold dilution with water to 0.8 % TFA, digestion was done with immobilized pepsin (Pierce), and samples were analyzed with MALDI mass spectroscopy. Varying digestion time shown that essentially complete fragmentation had occurred by 10 minutes, and samples from this time were used for peptide

assignment. A total of 14 peptic peptides were identified, 11 were readily assignable by their exact mass alone. The remaining 3 were assigned using CID. For completeness, the 11 peptide sequences were confirmed by CID as well. The 14 peptides represent 100 % sequence coverage of the 55-mer. Apparently, the C-terminal region is more readily cleaved. Further optimization of the digestion conditions, or addition of a second protease, may generate more N-terminal peptides.

Figure to the right:
Sequence of PrP(89-143)
and peptide map of the
pepsin digest of PrP 89-
143 P101L. Peptic
fragments are shown as
bars.

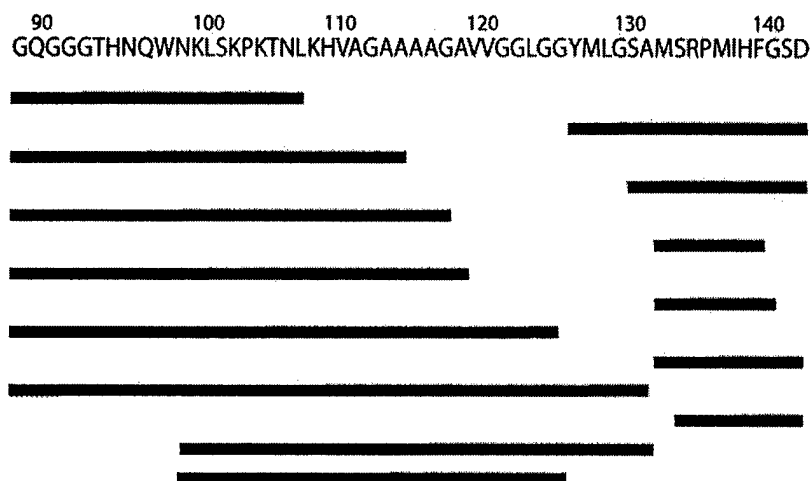
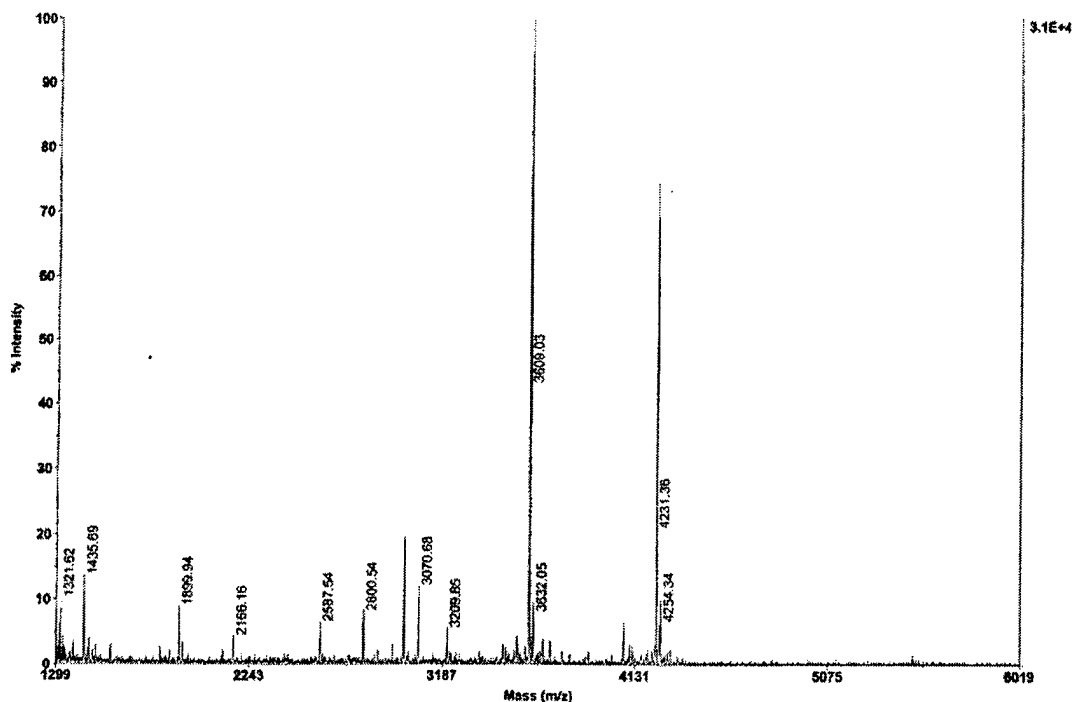
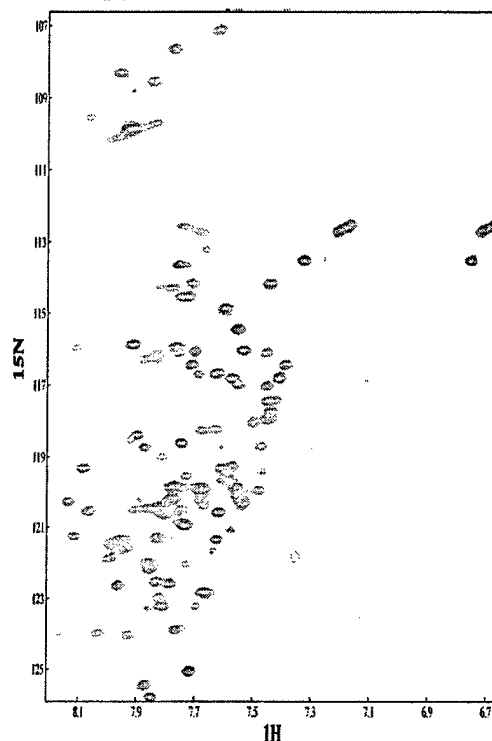


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nute pepsin digest of PrP indicating clean, high quality data can be obtained.

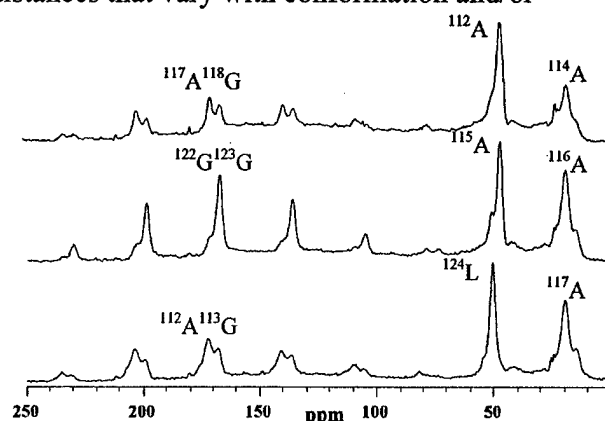
Since the amount of sample for P₅₅ is somewhat limited, and we do not yet have fully isotope labeled samples, we have also done a few test experiments with a small amyloid forming protein, TTR. The methodology used with it is directly applicable to PrP P₅₅ when more samples are available. Some mass spectroscopy detected hydrogen exchange experiments have also been done with TTR, showing that we can in fact localize regions protected from exchange (*i.e.* hydrogen bonded). The TTR experiments were done with Prof. V. Woods at UCSD, with a more automated system than that at Berkeley. While we have less access to the UCSD instrumentation, in the long run once conditions are established doing 'production' runs there should yield more data more quickly. We also did some "NMR detected" hydrogen exchange measurements with TTR, using uniform ¹⁵N labeling (available because we expressed TTR in *E.coli* at high levels some time ago). After some manipulation of conditions we showed that we could collect ¹H-¹⁵N correlation spectra (HSQC) in DMSO solvent (to quench exchange, example spectrum shown to the right). To interpret exchange data we need to assign these 'denatured' spectra in DMSO, but has been done for comparable size proteins previously, and should be easier for labeled P₅₅ it becomes available.



¹H-¹⁵N correlation (HSQC) spectrum of TTR (127 residues) in DMSO solution.

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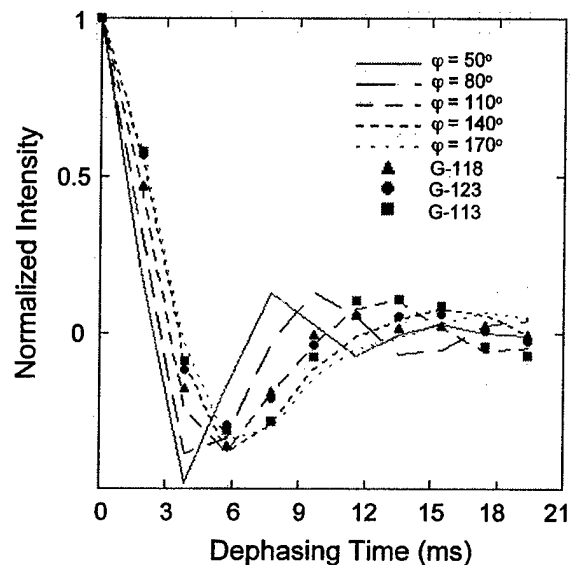
In solid state NMR experiments there are several ways to probe conformation, measuring either backbone angles at specific residues, or distances that vary with conformation and/or intermolecular packing. For most residues the backbone dihedral angles phi and psi can be determined from the isotropic chemical shift, or somewhat more accurately by measuring the shift anisotropy. However for glycine residues the lack of a side chain leaves ambiguity in the angles. Since recent models for the aggregated state of PrP have considered beta-helices, in which glycine can play a special role by occupying sites that are sterically crowded, it seems important to be



Solid state magic angle spinning (MAS) spectra of PrP(89-143)P101L peptides selectively labeled with ¹³C in successive carbonyls, and in alpha (50 ppm) or beta (20 ppm) positions of other amino acids as indicated.

able to probe glycine as well as the other amino acids. To do this a peptides were made in which the carbonyl groups of two successive amino acids were ¹³C labeled.

The relative orientations of these carbonyls were probed using DQ magic angle correlation spectra, and also by using double quantum filtered dephasing. A sample of spectra and dephasing curves are shown. With these data it appears that none of the glycines in the 112-123 segment is involved in a sharp turn, but G113 may not be in a fully extended beta-sheet conformation. The data do not provide positive evidence for a beta-helix, but have not extended over a sufficient number of residues to rule it out either. Further probing of the conformation with solid state NMR will be possible once a greater variety of labeled peptides can be made.



Work will continue as described in the original proposal, in particular continuing work to produce specifically isotope labeled P₅₅ using biological synthesis (both *in vitro* translation and expression in *E.coli*). The samples made will be used for further solid state NMR to define the conformation of the peptide in the fibril state. We will also move forward with deuterium exchange into fibrils of P₅₅ using the conditions we have defined for analysis. We will expand coverage by investigating other proteases to increase fragmentation, as has been done in Woods' group at UCSD. The only new aspect of work will be the addition of one new variation on PrP samples. Prusiner's group at UCSF has shown recently that expressed PrP(89-231) can be aggregated into fibrils that show biological activity [a paper is in press], extending their initial work with the 89-143 peptide. In addition to continuing our work with 89-143 (which may be better for the solid state NMR experiments due to the smaller size and hence simpler spectra), we will initiate experiments with PrP 89-231 to complement this. Since 89-231 has been expressed at high levels, labeling in *E.coli* will be possible, but the sample characteristics remain to be determined. We will also initiate hydrogen exchange experiments on fibrils of PrP₈₉₋₂₃₁ to compare with P₅₅, and ultimately with *in vivo* made PrP^{Sc}.

KEY RESEARCH ACCOMPLISHMENTS

- constructed gene for expression of PrP(89-143) carrying P101L mutation
- initiated expression trials (successful in *E.coli*) and *in vitro* translation (not yet successful)
- established conditions for rapidly dissolving PrP(89-143) fibrils and fragmenting for mass spectral analysis
- have established peptide identifications for peptide fragments in MALDI spectra for hydrogen exchange localization
- implemented rapid dissolution of test fibrils of TTR in organic solvent for NMR analysis of hydrogen exchange
- carried out solid state NMR studies of specifically labeled PrP(89-143) probing conformations in the residue 112-123 segment; the significant finding is that glycine residues are not found in right handed helical conformations that might have been expected for beta helix.

REPORTABLE OUTCOMES

A manuscript describing the solid state NMR experiments is in the final stages of revision, to be submitted to JACS.

A dissertation fellowship application was submitted to the Ford Foundation by Mr. Steven Damo associated with work on this project, and was funded.

CONCLUSIONS

Significant progress has been made implementing methods necessary for completion of the proposed research. Constructs have been made so that *in vitro* translation of PrP(89-143) can be tested, and also so that *in vivo* expression in *E.coli* can be done. These will enable isotope labeling for solid state NMR in ways that have not been feasible using peptide synthesis. Methods and conditions have been established for following exchange of backbone amide protons in fibrillar peptides using mass spectroscopy. Conditions to localize using fragments have been established, so that we are now in a position to proceed with real studies of exchange in the fibrils. Some solid state NMR studies have been done expanding the number of residues at which the local conformation has been probed. Significantly the conformations at three glycine residues were studied. These residues were of particular interest because they are more likely to be in turn conformations if the peptides form beta-helices as has been recently proposed as a model [3]. We did not find evidence for these turns.

Defining the conformation of PrP in 'scrapie-like' fibril states will help to understand the basis for the 'scrapie' diseases. The studies on fibrils will lead to testable hypothesis about full length PrP^{Sc} as it occurs *in vivo* and about the relationship between protein sequence and susceptibility to disease. The exchange studies will help define the conformation in the fibrils, and can be extended to studies of real PrP^{Sc} because only small amounts of material are required and the selectivity in the mass spectra allows analysis of complex mixtures much more effectively than other methods. The combination of approaches allows a correlation from the *in vitro* studies to the real systems *in vivo*.

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